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Insights into the Biogeography and Polyploid Evolution of New Zealand *Asplenium* from Chloroplast DNA Sequence Data

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ABSTRACT.—Nucleotide sequences of the chloroplast *trnL-trnF* intergenic spacer were obtained for 21 of the 22 indigenous *Asplenium* taxa presently recognized from New Zealand. Nucleotide sequences of the chloroplast *rbcL* gene were also obtained from eleven New Zealand species representative of the diversity found in the *trnL-trnF* intergenic spacer. Phylogenetic analyses of these chloroplast sequence data indicate that the *Asplenium* species of New Zealand are not monophyletic. More specifically, the *Asplenium* species participating in hybridization in New Zealand form a closely related 'Austral' group, whereas the non-hybridizing species have closer affinities to species from outside New Zealand. Within the Austral group, three well-supported sub-groups are recognized, represented by the species *A. bulbiferum*, *A. flaccidum*, and *A. obtusatum*. Dating analyses reject an 80 million year old vicariant origin for any of the *Asplenium* lineages in New Zealand, and the distributions of the many *Asplenium* species disjunct between New Zealand and elsewhere appear best explained by long-distance dispersal. The likely chloroplast/maternal parent for each of the New Zealand octoploid species is discussed.

Asplenium, with approximately 700 species worldwide, appears to have a complex history of hybridization and auto- and allopolyploidy, and is a model group for the study of fern evolution (e.g., Manton, 1950; Wagner, 1954; Lovis, 1977; Reichstein, 1981). Recent DNA sequencing studies (e.g., Murakami *et al.*, 1999a; Yatebe *et al.*, 2001; Gastony and Johnson, 2001; Pinter *et al.*, 2002; Van den heede *et al.*, 2002, 2003) have shed light on the evolutionary history of this iconic group of ferns. Here we provide chloroplast DNA sequences for 21 of the 22 indigenous *Asplenium* taxa in New Zealand (Table 1; Brownsey and Smith-Dodsworth, 2000; Brownsey, 2003), where the genus is often ecologically conspicuous and comprises about 10% of the indigenous fern flora.

Early taxonomic studies of New Zealand *Asplenium* (e.g., Hooker, 1855; Martin, 1920) indicated hybridization was rife and found the different 'kinds' to grade together. In his account of New Zealand *Asplenium*, Allan (1961, p. 75) remarked that the species were "very ill-defined", and that while many species appeared to respond "markedly to environmental conditions . . . [,] there is also

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TABLE 1. Indigenous New Zealand *Asplenium*, together with additional species included in study. Ploidy levels obtained from Löve *et al.* (1977), Braithwaite (1986), Murakami *et al.* (1999b), Dawson *et al.* (2000), Pinter *et al.* (2002), Tindale and Roy (2002), and Van den heede *et al.* (2003). Alternative taxonomy, in brackets, taken from Ogle (1987). Distribution details were compiled from various sources and, like ploidy level(s), will be dependent on taxon circumscriptions. Sample localities are only provided for New Zealand taxa, of which duplicate samples are identified by superscript letters. Herbarium vouchers are given for samples from which novel sequence data was generated for this study. Sequences obtained from GenBank are referenced by the study that generated them. GenBank accession numbers are given for the *trnL-trnF* intergenic spacer and *rbcL* gene sequences included in the analyses.

Taxon	Ploidy	Indigenous distribution: collection location for New Zealand samples	Herbarium voucher/ previous study	*trnL-trnF* intergenic spacer	*rbcL* gene
A. appendiculatum (Labill.) C.Presl subsp. *appendiculatum* [*A. terrestre* Brownsey subsp. *terrestre*]	8*x*	Australia, New Zealand: Mt. Cook	WELT P20492	AY283202	
A. appendiculatum subsp. *maritimum* (Brownsey) Brownsey [*A. terrestre* subsp. *maritimum* Brownsey]	8*x*	New Zealand endemic: Wellington	WELT P20493	AY283203	
A. bulbiferum G.Forst subsp. *bulbiferum* (*A. bulbiferum* G.Forst., *s.s.*)	4*x*	New Zealand endemic: [A]Palmerston North, [B]Stewart I.	[A]WELT P20494 [B]WELT P20495	[A,B]AY283204	[A]AY283226
A. bulbiferum subsp. *gracillimum* (Colenso) Brownsey (*A. gracillimum* Colenso)	8*x*	Australia, New Zealand: [A]Hunterville, [B]Stewart I.	[A]WELT P20496 [B]WELT P20497	[A]AY283205 [B]AY283206	
A. chathamense Brownsey	4*x*	New Zealand endemic: Chatham I.	WELT P20498	AY283207	
A. cimmeriorum Brownsey et de Lange	8*x*	New Zealand endemic: Punakaiki	WELT P20499	AY283208	
A. flabellifolium Cav.	4*x*, 5*x*, 6*x*, 8*x*, 12*x*	Australia, New Zealand: Dannevirke	WELT P20500	AY283209	AY283227
A. flaccidum G.Forst subsp. *flaccidum* (*A. flaccidum* G.Forst *s.s.*)	4*x*	Australia, New Zealand: [A]Dunedin, [B]Paihia	[A]WELT P20501 [B]WELT P20502	[A,B]AY283210	[A]AY283228
A. flaccidum subsp. *haurakiense* Brownsey (*A. haurakiense* (Brownsey) Ogle)	4*x*	New Zealand endemic: cultivated Auckland	WELT P20503	AY283211	

TABLE 1. Continued.

Taxon	Ploidy	Indigenous distribution: collection location for New Zealand samples	Herbarium voucher/ previous study	*trnL-trnF* intergenic spacer	*rbcL* gene
A. hookerianum Colenso	4*x*	Australia, New Zealand: [A]Dannevirke, [B]Banks Peninsula	[A]WELT P20504 [B]WELT P20505	[A]AY283212 [B]AY283213	[A]AY283229
A. lamprophyllum Carse	4*x*	New Zealand endemic: Auckland	WELT P20506	AY283214	AY283230
A. lyallii (Hook.f.) T.Moore	8*x*	New Zealand endemic: Castlepoint	WELT P20507	AY283215	
A. oblongifolium Colenso	4*x*	New Zealand endemic: [A]Paihia, [B]Palmerston North	[A]WELT P20508 [B]WELT P20509	[A,B]AY283216	[A,B]AY28323
A. obtusatum G.Forst subsp. *obtusatum* (*A. obtusatum* G.Forst., *s.s.*)	4*x*	New Zealand, South America: [A]Bluff, [B]Haast	[A]WELT P20510 [B]WELT P20511	[A,B]AY283217	[A,B]AY28323
A. obtusatum subsp. *northlandicum* Brownsey (*A. northlandicum* (Brownsey) Ogle)	8*x*	Australia, New Zealand, Pacific Islands: Auckland	WELT P20512	AY283218	
A. paupereequitum Brownsey et P.J.Jacks.	8*x*	New Zealand endemic: Poor Knights Is.	WELT P20513	AY283219	AY283233
A. polyodon G.Forst.	4*x*	Asia, Australia, New Zealand, Pacific Islands: Palmerston North	WELT P20514	AY283220	AY283234
A. richardii (Hook.f.) Hook.f.	8*x*	New Zealand endemic: Mt. Cook	WELT P20515	AY283221	
A. scleroprium Hombr.	8*x*	New Zealand endemic: Bluff	WELT P20516	AY283222	
A. shuttleworthianum Kunze	8*x*	New Zealand, Pacific Islands: cultivated Auckland (ex. Kermadec Is.)	WELT P20517	AY283223	AY283235
A. trichomanes L. subsp. *quadrivalens* D.E.Mey emend. Lovis	4*x*	Sub-cosmopolitian: not available			
A. trichomanes L. subsp. nov.	6*x*	Australia, New Zealand: Takaka	WELT P20518	AY283224	AY283236
A. aethiopicum (Burm.f.) Bech.	4*x*, 6*x*, 8*x*, 10*x*, 12*x*	Africa, Asia, Australia	Vogel unpublished/ Pinter *et al.*, 2002	AF240666	AF240654
A. antiquum Makino	4*x*	Asia	Yatabe *et al.* 2001		AB023502
A. aureum Cav.	4*x*	Macaronesia	Vogel unpublished	AF240657	AF240642

TABLE 1. Continued.

Taxon	Ploidy	Indigenous distribution: collection location for New Zealand samples	Herbarium voucher/ previous study	*trnL-trnF* intergenic spacer	*rbcL* gene
A. austraiasicum (J.Sm) Hook.	4*x*	Australia, New Guniea, Pacific Island	WELT P20519	AY283225	AY283237
A. ceterach L.	2*x*, 4*x*, 6*x*	Africa, Asia, Europe	Vogel unpublished	AF240658	AF240643
A. ensiforme Hook. et Grev.	4*x*	Asia	Murakami *et al.*, 1999a		AB014709
A. hemionitis L.	?	Africa, Europe	Pinter *et al.*, 2002	AF240663	AF240648
A. marinum L.	2*x*	Africa, Europe	Pinter *et al.*, 2002	AF240662	AF240647
A. nidus L.	4*x*	Asia, Pacific Islands	Yatabe *et al.*, 2001		AB023500
A. phillipsianum (Kümmerle) Bir, Fraser-Jenk. & Lovis	2*x*, 4*x*, 6*x*	Africa	Listed as *A. cordatum* (Thumb.) Sw. by Pinter *et al.* (2002), but group with sequences identified as *A. phillipsianum* by Van den heede *et al.* (2003).	AF525235	AF240650
A. prolongatum Hook	?	Asia	Murakami *et al.*, 1999a		AB014691
A. sagittatum (DC.) Bange	2*x*, 4*x*	Asia, Europe	Vogel unpublished/ Pinter *et al.*, 2002	AF240661	AF240646
A. setoi N. Murak. et Seriz	4*x*	Asia	Murakami *et al.*, 1999b		AB013243
A. theciferum (Kunth) Mett.	4*x*	Africa, America	Gastony and Johnson, 2001		AF336099
A. viride Huds.	2*x*	Africa, Asia, Europe, North America	Pinter *et al.*, 2002	AF240664	AF240649
A. wrightii Hook.	8*x*	Asia	Murakami *et al.*, 1999a		AB014690
Hymenasplenium unilaterale (Lam.) Hayata	2*x*, ?4*x*	Africa, Asia, Australia, Pacific Islands	Vogel unpublished/ Pinter *et al.*, 2002	AF240668	AF240652

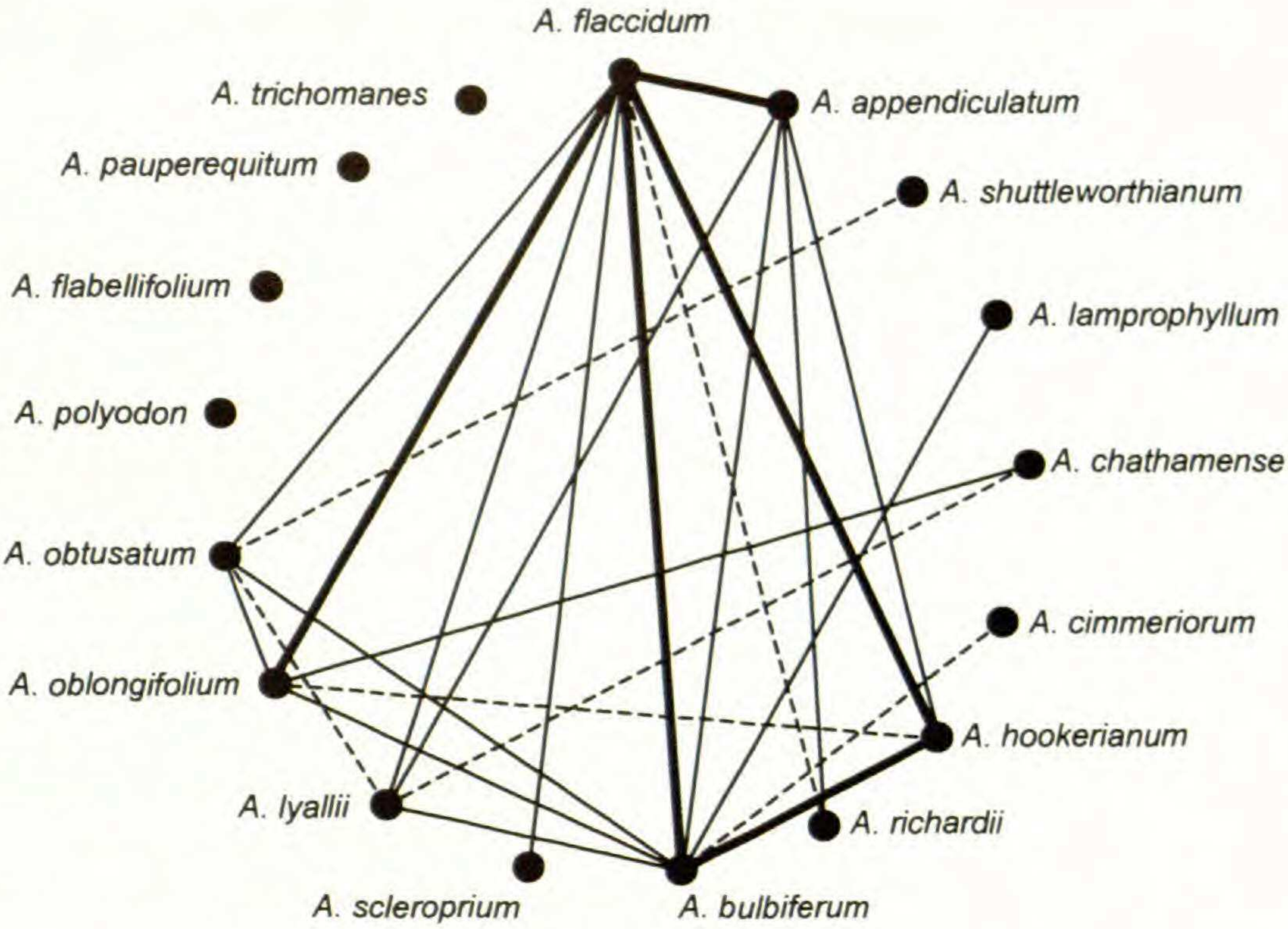

FIG. 1. Crossing polygon showing the *Asplenium* hybrid combinations known to occur naturally in New Zealand, updated from Brownsey (1977b, fig. 23). Thick line, intermediate line, dashed line, and no line, indicates hybridization is common, intermediate, rare, and unknown, respectively. Relative frequencies determined from analysis of 190 hybrid specimens cited by Brownsey (1977b) and more recent collections in WELT. Subspecific taxa are not distinguished in the polygon because it is often difficult to determine which subspecies contributed to a hybrid.

no doubt that hybridism plays an important part''. However, Brownsey (1977a, 1977b) concluded that while hybridization amongst the species of New Zealand *Asplenium* was indeed relatively common, the spores of the hybrid plants were aborted and introgressant swarms were virtually absent.

Brownsey (1977b) also noted that hybridization was unequally distributed, with some taxa participating frequently and others not at all. Although geography and ecology influence the frequency of hybridisation, some taxa are not known to form hybrids in New Zealand despite frequent sympatric occurrence with congenerics. For instance, *Asplenium polyodon*, despite frequent sympatry, is not known to hybridize with *A. bulbiferum* subsp. *bulbiferum*, *A. bulbiferum* subsp. *gracillimum*, *A. flaccidum* subsp. *flaccidum*, or *A. oblongifolium*. Similarly, *A. trichomanes*, despite frequent sympatry, is not known to hybridize with *A. bulbiferum* subsp. *gracillimum*, *A. hookerianum*, or *A. lyallii*.

A crossing diagram (Fig. 1) shows the extent and frequency of hybridization amongst New Zealand *Asplenium* taxa. Brownsey (1977b) suggested that the *Asplenium* taxa involved in hybridization in New Zealand were more closely related to one another, and constituted an 'Austral' group of *Asplenium*. Species from this Austral group, comprising *A. appendiculatum*, *A. bulbiferum*,

A. chathamense, *A. cimmeriorum*, *A. flaccidum*, *A. hookerianum*, *A. lamprophyllum*, *A. lyallii*, *A. oblongifolium*, *A. obtusatum*, *A. richardii*, *A. scleroprium* and *A. shuttleworthianum*, are generally common in New Zealand, and some of these species or their close relatives also occur in southern Australia, the southern Pacific, and South America. There are no diploid *Asplenium* species in New Zealand (Dawson *et al.*, 2000), but amongst the tetraploid New Zealand representatives of the Austral *Asplenium* group, Brownsey (1977a, p.84) suggested the presence of "three natural groups of closely related species . . . : (1) the *A. flaccidum* aggregate with thick and rather leathery bipinnate fronds, (2) the *A. hookerianum/bulbiferum* complex with thin highly dissected fronds, and (3) the *A. lucidum/obtusatum* complex with thick fleshy pinnate fronds" (*Asplenium lucidum* G.Forst. non N.L.Burman is an earlier, but illegitimate, name for *A. oblongifolium* Colenso; Brownsey, 1979).

Brownsey (1977b) considered the *Asplenium* species not hybridizing in New Zealand to have closer affinities with groups other than the Austral group. For instance, *A. polyodon* is widespread in the palaeo-tropics and has morphological similarities to species such as *A. cuneatum* and *A. aethiopicum*. *Asplenium trichomanes* is widespread in the northern hemisphere. In Europe, diploid, tetraploid and hexaploid forms of this species are known, and there is good cytological and morphological evidence that these hybridize naturally with at least 13 other distinct species (Reichstein, 1981), in contrast to the total absence of hybridization in New Zealand. *Asplenium flabellifolium* also appears to have northern hemisphere affinities, showing some morphological resemblance to *A. viride*. The relationship of *A. pauperequitum* is less clear. Brownsey and Jackson (1984) suggested that, based on morphology, this endemic species was not closely related to any of the other New Zealand species, although it may have distant affinities with *A. polyodon*.

This mixture of postulated affinities suggests that the extant *Asplenium* taxa in New Zealand do not share a common New Zealand ancestor and that they are not monophyletic. Rather, it suggests they are derived from several non-New Zealand ancestors, implicating multiple, distinct origins of *Asplenium* in New Zealand. If multiple origins for New Zealand *Asplenium* are indeed the case, then of considerable biogeographic interest is when these lineages arrived. Are the separate lineages of New Zealand *Asplenium* old, perhaps in accord with the separation of New Zealand from the remainder of Gondwana about 80 million years ago (McLoughlin, 2001), and therefore consistent with a vicariant explanation? Or, do they appear younger than 80 m.y.a., necessitating a long-distance dispersal explanation for their origins?

Amongst the New Zealand members of the Austral *Asplenium* group there are eight tetraploid and nine octoploid taxa. Five of these octoploids are endemic to New Zealand, and from morphological and ecological evidence they have been hypothesized to have arisen via auto- or allopolyploidy (Brownsey, 1977b; Brownsey & de Lange, 1997).

To conduct a phylogenetic analysis of the New Zealand *Asplenium* and to test whether their relationships are indeed reflected in hybridization frequency/ability, we obtained DNA sequence data from two chloroplast markers. While

the chloroplast may only be inherited maternally, as has been reported by Vogel *et al.* (1998) for European *A. trichomanes*, we anticipated that a chloroplast phylogeny of New Zealand *Asplenium* would nevertheless provide important insights into their origins, in terms of both the number of origins and, via a molecular clock approach, their timing. We also hoped to identify the chloroplast, or maternal, parent for each of the Austral octoploids.

The *trnL-trnF* intergenic spacer was sequenced for every New Zealand *Asplenium* taxon presently recognized (Table 1), except *A. trichomanes* subsp. *quadrivalens* for which we know of no extant population in New Zealand. We did not include in our study *Pleurosorus rutifolius* (R.Br.) Fée, another member of the Aspleniaceae indigenous to New Zealand, but its phylogenetic position is currently being investigated elsewhere (Johannes Vogel, pers. comm.). Not only does the *trnL-trnF* intergenic spacer amplify easily and consistently across a wide range of plants but, because it is non-coding, it evolves relatively fast, and accumulates both base-pair substitutions and insertion-deletion ('indel') events that may be phylogenetically informative. It is therefore one of the best sequence markers available for detecting genetic differences amongst the New Zealand *Asplenium* taxa.

The *rbcL* gene was sequenced for representative New Zealand *Asplenium* taxa. Although the *rbcL* gene may evolve slower than the *trnL-trnF* intergenic spacer, it does not accumulate indel events, which means the alignment of sequences from different taxa is easier. In addition, the large existing database of *rbcL* sequences for *Asplenium* taxa from around the world allows placement of representative New Zealand taxa into a global framework.

Materials and Methods

Table 1 details for each of the indigenous *Asplenium* taxa currently recognized from New Zealand (Brownsey and Smith-Dodsworth, 2000; Brownsey, 2003) the taxonomy adopted for this study, the natural distribution, ploidy level, and the location of the sample(s) analyzed. We note that for some of these taxa there is debate as to whether they should be recognized at the subspecifc (Brownsey, 1977a) or specific (Ogle, 1987) level, and provide the alternative taxonomy where appropriate. Herbarium abbreviations follow Holmgren *et al.* (1990).

DNA was extracted from silica-gel dried tissue using a modified CTAB protocol (Doyle & Doyle, 1990). PCR was performed in 20 μL volumes containing 1× Q solution (Qiagen), 10mM Tris-HCl pH 8.8, 50 mM KCl, 1.5mM $MgCl_2$, 250 μmol dNTPs, 10 ρmol of each primer, 1 U of Taq DNA polymerase (Qiagen), and approximately 50 ng of template DNA.

The complete sequence for the *trnL-trnF* intergenic spacer was obtained from every *Asplenium* taxon indigenous to New Zealand (except *Asplenium trichomanes* subsp. *quadrivalens*; Table 1) using the primers E and F of Taberlet *et al.* (1991) and a thermocycling profile beginning with an initial denaturation of 94°C for 2 minutes, followed by 38 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for

5 minutes. Duplicate samples of the geographically-widespread tetraploid taxa in New Zealand (*A. bulbiferum* subsp. *bulbiferum*, *A. hookerianum*, *A. flaccidum* subsp. *flaccidum*, *A. oblongifolium*, and *A. obtusatum* subsp. *obtusatum*), which are the most likely to be genetically variable, were analyzed to assess infra-taxon variation in this marker. Duplicate samples of the widespread octoploid *A. bulbiferum* subsp. *gracillimum* were also analyzed. Locality details, herbarium voucher number, and GenBank accession numbers are given in Table 1. Sequence for the *trnL-trnF* intergenic spacer was also obtained from a cultivated plant of *A. australasicum* (J.Sm.) Hook. (Table 1).

Sequences for the *rbcL* gene were obtained for representative New Zealand species from each of the three Austral groups, each of the non-Austral New Zealand species, and a cultivated plant of *Asplenium australasicum* (Table 1). The external primers aF and cR of Hasebe *et al.* (1994) were used with a thermocycling profile beginning with an initial denaturation of 94°C for 2 minutes, followed by 38 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. The novel internal primers rbcLAsForward2 (5′-AAGCCAAAATTAGGTCTATCTGC-3′) and rbcLAsReverse2 (5′-CCCAATTCTCTCGCAAAAACAG-3′) were used where necessary to obtain single amplification products and/or complete bi-directional sequencing.

PCR products were purified using the CONCERT Rapid PCR Purification System (Gibco BRL). The purified PCR products were sequenced in both directions using an Applied Biosystems 373A DNA Sequencing System and the ABI PRISM™ Dye Terminate Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

Additional *trnL-trnF* and *rbcL* sequences were obtained from GenBank for species representative of the genetic diversity previously reported in the Aspleniaceae (Murakami *et al.*, 1999a; Yatebe *et al.*, 2001; Gastony and Johnson, 2001; Pinter *et al.*, 2002; Van den heede *et al.*, 2003). Details are provided in Table 1. Only *rbcL* sequence was available for some of the selected species, preventing their inclusion in the *trnL-trnF* alone and combined analyses (see below).

In this study we follow Murakami (1995) in the recognition of *Hymenasplenium* Hayata as a separate genus sister to *Asplenium*, and use *H. unilaterale*, for which both *trnL-trnF* and *rbcL* sequences are available from GenBank, as the outgroup. However, to test the effect of outgroup choice (Adachi and Hasegawa, 1995), the *rbcL* analyses were repeated with *H. unilaterale* replaced with either *H. hondoense* (N. Murak. et Hatanaka) Nakaike (GenBank ABO14705) or *H. riparium* (Liebm.) N. Murak. (ABO14708). Previous studies (e.g., Murakami *et al.*, 1999a; Van den heede *et al.*, 2003) indicate these species are representative of the diversity known within *Hymenasplenium*.

The sequences for each marker were aligned with ClustalX 1.8 (Thompson *et al.*, 1997). The alignment of indels amongst the *trnL-trnF* sequences was edited further with Se-Al v1.0 (Rambaut, 1995). One central region of the *trnL-trnF* intergenic spacer could not be unambiguously aligned between the {New Zealand Austral *Asplenium* species + *A. australasicum*} and the remaining

species. It was excluded from all analyses except those encompassing only the New Zealand Austral species and *A. australasicum*. Analyses of the *trnL-trnF* sequences were performed with sites encompassing indels either completely excluded, or included and treated as missing data. In addition, analyses were also performed with the large indels of *A. marinum*, *A. aethiopicum*, *A. trichomanes*, *A. hemionitis*, and the *A. flaccidum* group (the MATHF indels) included and treated as missing data, but with all other indels excluded.

PAUP* 4.b10 (Swofford, 2002) was used to perform heuristic search analyses under maximum parsimony (MP; with tree bisection-reconnection branch-swapping, and 100 replicates of random sequence addition), and maximum likelihood (ML; with tree bisection-reconnection branch-swapping, and random sequence addition). Appropriate models of evolution for ML were selected using Modeltest v3.06 (Posada and Crandall, 1998) with the hierarchical likelihood-ratio test. Analyses were conducted on the *trnL-trnF* and *rbcL* data sets separately, and with them combined into a single data set after testing for incongruence using a partition homogeneity test as implemented in PAUP* 4.b10. Bootstrapping was performed to assess confidence in the groups identified by maximum parsimony (n = 1000) or maximum likelihood (n = 100; with the parameters estimated by Modeltest fixed).

The timing of divergences within *Asplenium* were estimated with the program r8s v.1.60 (Sanderson, 2002), which offers methods with the advantage of allowing rates of evolution to vary throughout the tree, and has been applied to other pteridophyte groups (e.g., Lycopodiaceae, Wikström and Kenrick, 2001; *Equisetum*, Des Marais *et al.*, 2003). The tree used for the r8s analysis was that estimated by maximum likelihood. The r8s analysis was implemented under penalised likelihood, using the Powell algorithm with a log-scale penalty function, and a smoothing value of 125 as determined by cross-validation. Following the recommendation of Sanderson (2002), more distant outgroups (*Polystichum vestitum* (G. Forst.) C. Presl, GenBank AF208395; *Thelypteris acuminata* (Houtt.) Morton, D43919; *Grammitis tenella* Kaulf., AF468198) were used to estimate the branch length of *Hymenasplenium unilaterale* before being discarded prior to the r8s analysis. Confidence intervals (mean ± standard deviation) were calculated through a bootstrapping procedure with 100 replicates (Sanderson and Doyle, 2001). The analysis was calibrated temporally by assigning an age of 140 m.y. (million years) to the most recent common ancestor of what appears to be the most distantly related extant components of the Aspleniaceae, *Hymenasplenium* and *Asplenium*. This date follows Skog (2001) who reported Aspleniaceae fossils from the early Cretaceous.

Results

Only two instances of *trnL-trnF* sequence variation within a taxon were found. The two samples of *Asplenium bulbiferum* subsp. *gracillimum* differ at position 50. The two samples of *A. hookerianum* also vary at position 50, as well as at two other positions invariant in the other taxa investigated.

Because of insertions and deletions, the length of the *trnL-trnF* sequences ranged between 238 and 398 base pairs. The *trnL-trnF* alignment, containing indels, comprised 393 positions for the Austral group plus *Asplenium australasicum*, and 428 positions for all taxa with the central region that could not be unambiguously aligned excluded. Maximum parsimony analysis of all *trnL-trnF* sequences strongly supported recognition of an Austral clade, with *A. australasicum* recovered as its sister (not shown). As depicted in Fig. 2, MP analysis of the *trnL-trnF* sequences of the Austral taxa, together with *A. australasicum* as an outgroup, indicated three strongly supported sub-clades within the Austral clade, more or less corresponding to the groups suggested by Brownsey (1977a; see above).

The Flaccidum clade, with 98% bootstrap support (BS), includes *Asplenium appendiculatum* subsp. *appendiculatum*, *A. appendiculatum* subsp. *maritimum*, *A. chathamense*, *A. flaccidum* subsp. *flaccidum*, *A. flaccidum* subsp. *haurakiense*, *A. lamprophyllum*, and *A. shuttleworthianum*. The Bulbiferum clade, with 98% BS, includes *A. bulbiferum* subsp. *bulbiferum*, *A. bulbiferum* subsp. *gracillimum*, *A. cimmeriorum*, *A. hookerianum*, and *A. richardii*. The Obtusatum clade, with 100% BS, includes *A. lyallii*, *A. oblongifolium*, *A. obtusatum* subsp. *northlandicum*, *A. obtusatum* subsp. *obtusatum*, and *A. scleroprium*. The Bulbiferum and Flaccidum clades are supported as sister clades with 85% BS. The inclusion/exclusion of indel sites had minimal effect on these principal results.

Incorporation of representatives from each of the three Austral sub-clades in analyses of *rbcL* sequences with a broader sample set, including species for which *trnL-trnF* sequences were not available, also supported recognition of the Austral clade. The *rbcL* sequences of *A. oblongifolium* and *A. obtusatum* were identical, so only the former was included in the analyses. The alignment of the *rbcL* sequences, with no inference of indels necessary, comprised 1191 base pairs.

Using the model selected by Modeltest (TrN + I + G; base frequency = [0.2698, 0.2215, 0.2461, 0.2626]; rate matrix = [1, 6.1972, 1, 1, 11.4499]; Invariable sites proportion = 0.5235; Gamma shape parameter = 0.8450), ML analysis selected a single tree with a –ln likelihood score of 4426.3070. This is presented in Fig. 3, where bootstrap support is also indicated. Eight trees of 487 steps (CI = 0.624, RI = 0.689, RC = 0.430) are recovered under maximum parsimony. The consensus of these equally most parsimonious trees (not shown) is similar to the set of relationships depicted by the maximum likelihood tree, except that the position of *Asplenium antiquum* within the Greater-nidus group is unresolved, *A. hemionitis* and *A. phillipsianum* form a clade that is sister to the remainder of the Marinum group, and *A. wrightii* is part of the basal polytomy rather than sister to the remainder of *Asplenium*.

Four major, monophyletic groups within this set of *Asplenium* are evident, which for communication purposes we here label as the Aethiopicum, Ceterach-Phyllitis, Marinum, and Greater-nidus groups (Fig. 3). *Asplenium wrightii* does not appear to be closely related to any of these groups, and it may represent a fifth group within *Asplenium*. Support for these four major groups

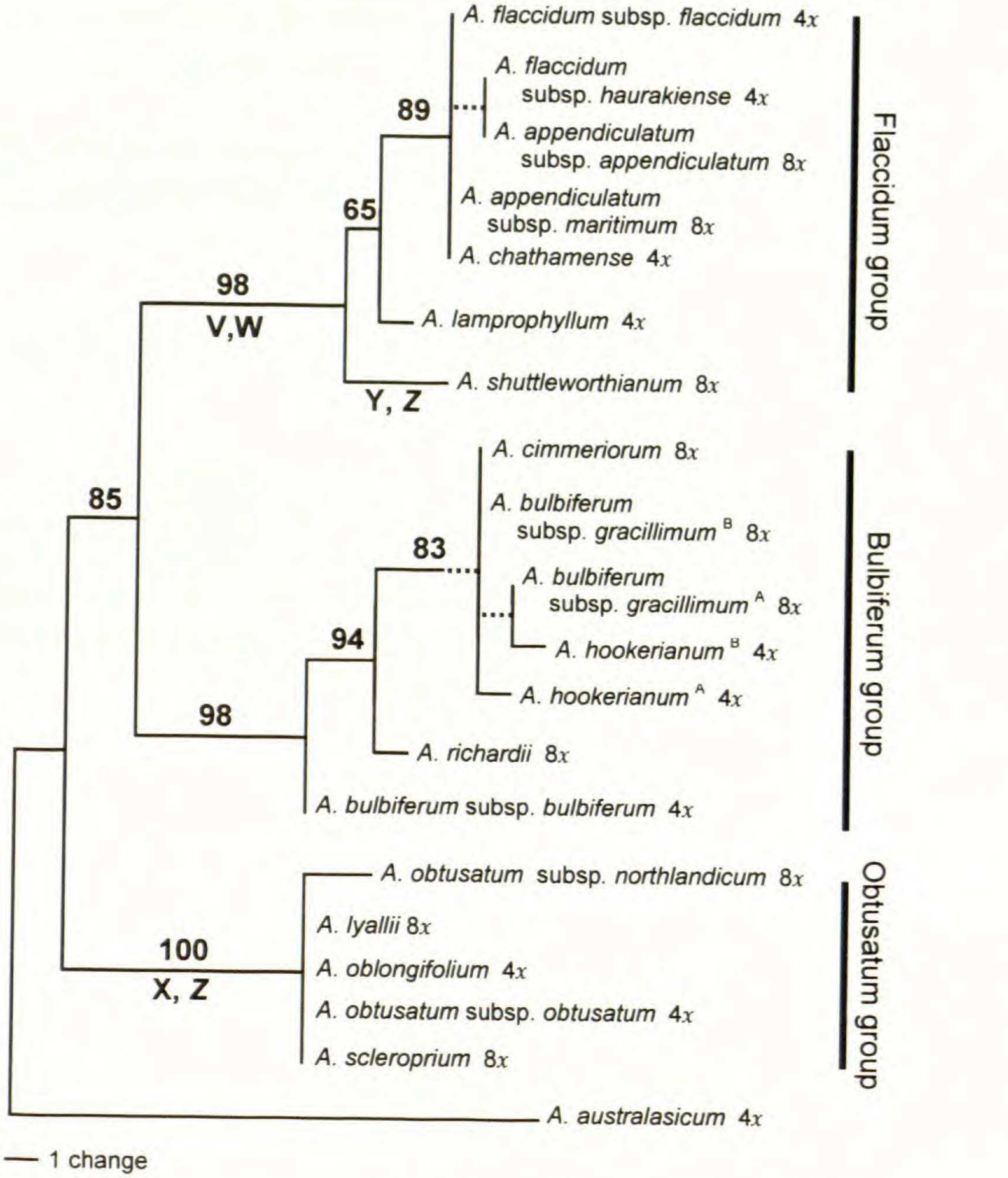

FIG. 2. Chloroplast phylogeny for all New Zealand taxa of the Austral group of *Asplenium*, from DNA sequences of the *trnL-trnF* intergenic spacer. *Asplenium australasicum* is the outgroup, and all indels were treated as missing data. One of three most equally parsimonious trees of 56 steps; the other two trees differ only in the arrangement of the *A. bulbiferum* subsp. *gracillimum*, *A. cimmeriorum*, and *A. hookerianum* samples. Ploidy level (Table 1) is listed after each taxon. Superscripts A and B indicate infra-taxon sequence variation, and correspond to the samples in Table 1. The position of indels within the Austral group is indicated by letters; italics indicate homoplastic indels: V, 2 base-pair (b.p.) deletion; W, 22 b.p. deletion; X, 2 b.p. insertion; Y, 5 b.p. deletion; *Z*, 1 b.p. deletion. Branches collapsing with the exclusion of base pair position 50 are shown as dashed lines. Bootstrap support is indicated where 50% or greater.

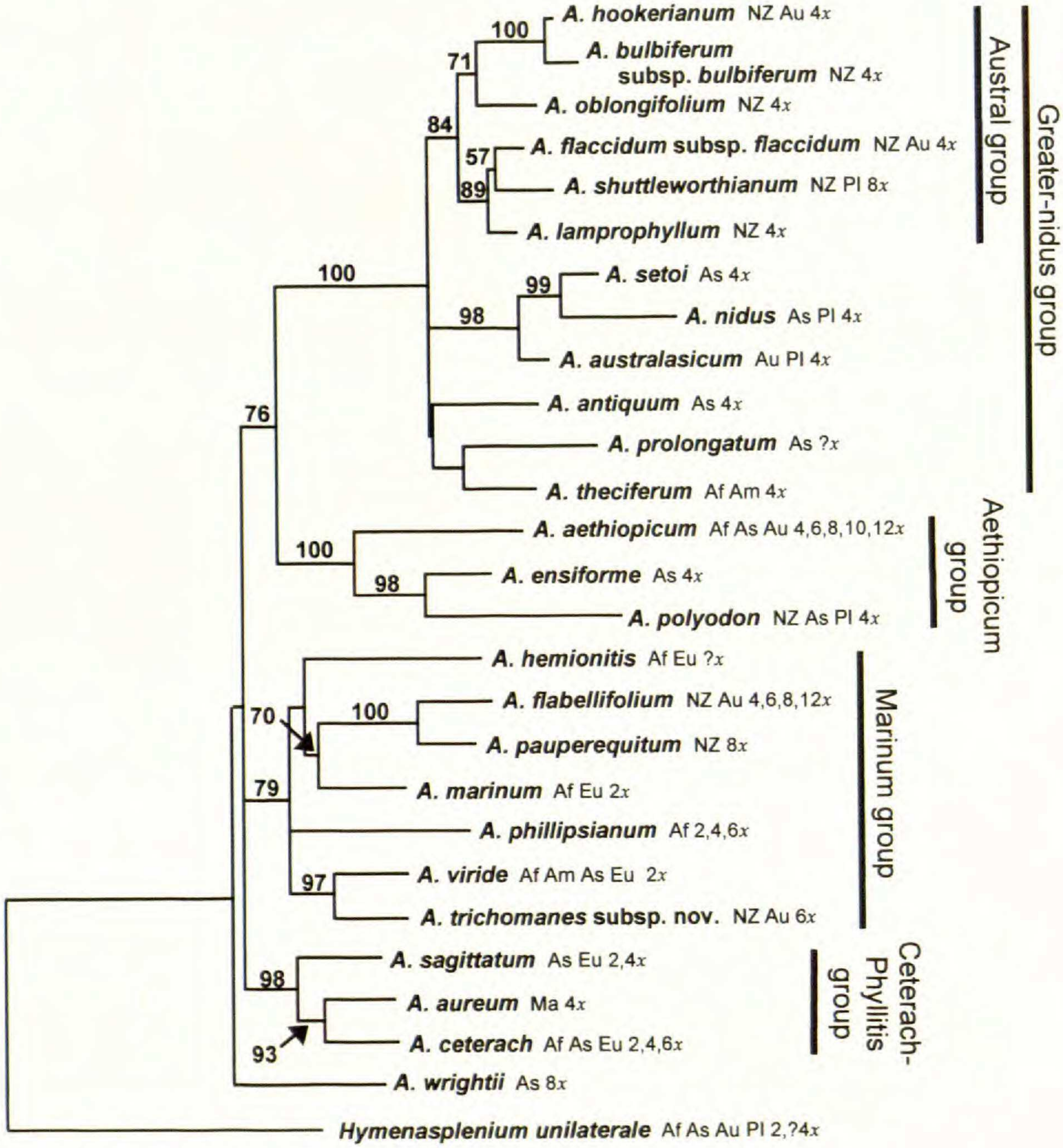

FIG. 3. Chloroplast phylogeny from maximum likelihood analysis of *rbcL* sequence data for representative *Asplenium* species: the tree with the best likelihood score, –ln L = 4426.3070. Bootstrap support is indicated above the corresponding branches. Distribution (Af, Africa; Am, North and South America; As, Asia; Au, Australia; Eu, Europe; Ma, Macaronesia; NZ, New Zealand; PI, Pacific Islands) and ploidy of species is shown (also see Table 1). Note that the *rbcL* sequence (and consequently position in the above tree) of *A. obtusatum* subsp. *obtusatum* is identical to that of *A. oblongifolium*.

is strong across different analyses (except for the Marinum group in some instances), but the relationships between them are not clear (see below). The Austral group is a sub-group within the Greater-nidus group, together with the birds' nest ferns *A. antiquum*, *A. australasicum*, *A. nidus*, and *A. setoi*, as well

as *A. theciferum* and *A. prolongatum*. The Greater-nidus group is supported with 100% BS under MP and ML. The Austral group is supported with about 80% BS under MP and ML. Within the Austral group, the Obtusatum (represented by *A. oblongifolium*) and Bulbiferum groups are supported as sister groups with about 70% BS under both MP and ML, which is in conflict with the *trnL-trnF* analysis. The species or group of species within the Greater-nidus group most closely related to the Austral group is not well resolved.

Of the New Zealand species not belonging to the Austral group, *Asplenium polyodon* falls within the Aethiopicum group, and *A. paupererequitum*, *A. flabellifolium*, and *A. trichomanes* within the Marinum group. *Asplenium flabellifolium* and *A. paupererequitum* are strongly supported as sister species whereas *A. trichomanes* appears closest, of the species sampled, to *A. viride*. Use of *Hymenasplenium hondoense* or *H. riparium* as the outgroup instead of *H. unilaterale* had little effect on these results, except in both cases BS for the Marinum group was less than 50% under MP.

Combination of the *rbcL* and *trnL-trnF* data sets is supported by a partition homogeneity test ($P = 0.14$). Analyses of this combined data set are consistent with those of the *rbcL* data set in that four major, monophyletic groups can be recognized within the *Asplenium* sample set analysed here, and that the Austral group is part of the Greater-nidus group. With the MATHF indel sites included and treated as missing data but all other indel sites excluded, five most equally parsimonious trees of 659 steps (CI = 0.716, RI = 0.712, RC = 0.510) are recovered (not shown). These differ in whether the Obtusatum group or the Flaccidum group diverge most basally within the Austral group, and whether the Aethiopicum group is sister to the Marinum group, an amalgamation of the Greater-nidus and Ceterach-Phyllitis groups, or to all three of these other major groups. Using the model selected by Modeltest (TIM + G; base frequency = [0.2777, 0.2162, 0.2367, 0.2694]; rate matrix = [1, 3.4608, 0.4752, 0.4752, 5.1292]; Gamma shape parameter = 0.3029), ML analysis selected a single tree with a –ln likelihood score of 5766.1590 (not shown). This has the Flaccidum group diverging most basally within the Austral group, and the Aethiopicum group sister to an amalgamation of the Greater-nidus and Ceterach-Phyllitis groups. In both instances the relationships are supported with less than 50% BS.

A difference between analyses of the data sets is the sister group of the Greater-nidus group. In the *rbcL* data it is the Aethiopicum group (61% BS MP, 76% BS ML), whereas in the combined *rbcL* and *trnL-trnF* data set it is the Ceterach-Phyllitis group (93% BS MP, 97% BS ML; the Greater-nidus and Ceterach-Phyllitis sister relationship is also recovered in analysis of the *trnL-trnF* data set alone). In addition, the bootstrap support for the Marinum group is lower in the combined *rbcL* and *trnL-trnF* data set (<50% BS MP, 56% BS ML) than the *rbcL* data set (70% BS MP, 79% BS ML). When all indel sites in the combined *rbcL* and *trnL-trnF* data set are excluded the four major groups are still recovered (not shown), albeit with the Marinum group with only low BS, but their relationships to each other are unresolved.

The age of the most recent common ancestor of the Greater-nidus group was calculated by r8s, under penalised likelihood with the Powell algorithm and

a log-scale penalty function, at 34 ± 5 million years ago (m.y.a.). Similarly, the ages calculated by r8s for the most recent common ancestor between each of the non-Austral New Zealand species and their closest non-New Zealand relative in this data set were: *Asplenium polyodon* and *A. ensiforme*, 31 ± 6 m.y.a.; {*A. flabellifolium* + *A. pauperequitum*} and *A. marinum*, 43 ± 7 m.y.a.; and *A. trichomanes* and *A. viride*, 30 ± 7 m.y.a. Substituting *H. hondoense* or *H. riparium* as the outgroup instead of *H. unilaterale* had little effect on these results, producing age estimates differing by only ±2 m.y.

The *trnL-trnF* sequences of the tetraploids *Asplenium flaccidum* subsp. *flaccidum* and *A. chathamense*, and the octoploid *A. appendiculatum* subsp. *maritimum* are identical to one another. The *trnL-trnF* sequences of the tetraploid *A. flaccidum* subsp. *haurakiense* and the octoploid *A. appendiculatum* subsp. *appendiculatum* are also identical, and differ from the three aforementioned taxa by a single shared base-pair substitution, at position 50. The *trnL-trnF* sequence of the octoploid *A. shuttleworthianum* differs from all others sampled by several base-pair substitutions and indel events.

The octoploids *Asplenium bulbiferum* subsp. *gracillimum* and *A. cimmeriorum* share several putative synapomorphies in their *trnL-trnF* sequence with the tetraploid *A. hookerianum*, and differ from the tetraploid *A. bulbiferum* subsp. *bulbiferum* by between five and seven base-pair substitutions. Depending on the reconstruction of character evolution, the octoploid *A. richardii* shares two or three *trnL-trnF* sequence putative synapomorphies with *A. hookerianum*, *A. bulbiferum* subsp. *gracillimum*, and *A. cimmeriorum*, but branches basally to them and is sister to that clade of species.

The *trnL-trnF* sequences of the octoploids *A. lyallii* and *A. scleroprium* are identical to those of the tetraploids *A. oblongifolium* and *A. obtusatum*, while that of the octoploid *A. obtusatum* subsp. *northlandicum* exhibits two autapomorphies.

Discussion

The evolutionary relationships inferred here from chloroplast sequences support the hypothesis based on hybridization frequency that New Zealand *Asplenium* are non-monophyletic, and that there is one large, relatively closely related 'Austral' group to which several other New Zealand species are only distantly related. Members of the Austral group hybridize with one another in New Zealand, but not with the non-Austral species. Similarly, Murakami *et al.* (1999a) observed natural hybridization to only occur between closely related *Asplenium* species.

Our analyses indicate at least four major monophyletic groups within the set of *Asplenium* investigated here, and our findings more or less correspond to those of other studies of chloroplast sequences (eg. Murakami *et al.*, 1999a; Gastony and Johnson, 2001; Pinter *et al.*, 2002). However, while the support in our analyses for each of these groups is generally strong (the Marinum group being an exception in some analyses), the relationships between them are not clear. This may be resolved by additional sampling, which may also indicate

the presence of other major monophyletic groups (e.g., *A. wrightii*?) or a re-circumscription of the groups outlined here.

Species from the Austral group have not previously been sampled genetically. Although Schulze *et al.* (2001) reported a *rbcL* sequence (GenBank AF318601) for a plant of *"A. bulbiferum"* from the Heidelberg Botanical Garden, their sequence is very different from that of the wild New Zealand material of *A. bulbiferum* reported here. Of the species here sampled, AF318601 appears closest to *A. theciferum* (not shown). The placement of the Austral group as a well-supported sub-group within the Greater-nidus group is perhaps unsuspected from morphological comparison.

The Greater-nidus group itself appears to have a predominantly southern hemisphere and north-western Pacific distribution, with *Asplenium prolongatum* also extending to India and China. Available chromosome counts (Löve *et al.*, 1977; Murakami *et al.*, 1999b; Dawson *et al.*, 2000; Tindale and Roy, 2002) indicate that all of the species sampled here from the Greater-nidus group are at least tetraploid, suggesting that this may be the ancestral state of the group. Further sampling and investigation is required to confirm this. Also of interest in the Greater-nidus group is the apparent lack of a close relationship between *A. antiquum* and the other birds' nest ferns, or between the Austral group and the other members of the Greater-nidus group with divided laminae (i.e., *A. prolongatum* and *A. theciferum*).

Even with this limited sampling, it is clear that New Zealand's *Asplenium* flora has had multiple independent origins from distantly related parts of the genus. There has been at least one separate migration in the Greater-nidus group, at least one in the Aethiopicum group, and at least two in the Marinum group. Moreover, molecular dating clearly indicates that these origins can be attributed to dispersal, and not vicariance. All age estimates for the most recent common ancestor of each New Zealand species and their closest non-New Zealand relative in this sample set are younger than 80 m.y.a., which is approximately when the New Zealand landmass separated from Australia and the rest of Gondwana. The closest age estimate to this boundary is the 43 m.y.a. between {*A. flabellifolium* + *A. pauperequitum*} and *A. marinum*. However, this is probably, at least in part, reflecting a distant relationship between *A. marinum* and *A. flabellifolium* or *A. pauperequitum*. In all cases, inclusion of non-New Zealand samples more closely related to each New Zealand *Asplenium* would make the age estimates younger. Also consistent with a dispersal interpretation for the origins of New Zealand *Asplenium*, Mildenhall (1980) gives the mid-Miocene (about 15 m.y.a) as the earliest appearance of *Asplenium* spores in the New Zealand fossil record.

Of considerable importance to the molecular dating is the use of an age of 140 m.y. for the most recent common ancestor of *Asplenium* and *Hymenasplenium* to calibrate the genetic divergences within *Asplenium*. If the separation between *Asplenium* and *Hymenasplenium* was actually older than 140 m.y., then the divergences estimated here within *Asplenium* would also be older. However, constraining the most recent common ancestor of the Greater-nidus group to 80 m.y.a., which is the youngest age consistent with a vicariant

origin of the New Zealand Austral *Asplenium* species amongst this data set, results in a calculation of 325 m.y.a. for the divergence between *Asplenium* and *Hymenasplenium*. This is quite inconsistent with the fern fossil record (Skog, 2001), indicating that even allowing for errors in the calibration, 80 m.y. old vicariant origins for New Zealand *Asplenium* can be rejected.

Alternatively, although the earliest known Aspleniaceae fossils may be approximately 140 m.y. old, the separation between *Asplenium* and *Hymenasplenium* could be more recent (and perhaps much more). If so, the divergences within *Asplenium* would also be younger than estimated here, but would still necessitate inference of dispersal rather than vicariance for the origins of New Zealand *Asplenium*. Indeed, an important caveat is that because the fossil record places, as far as we know, no 'younger' bound on the divergences investigated here, their real ages could be orders of magnitude younger than calculated.

Within the Austral group, the following taxa also occur outside New Zealand: *Asplenium appendiculatum* subsp. *appendiculatum*, *A. bulbiferum* subsp. *gracillimum*, *A. flaccidum* subsp. *flaccidum*, *A. hookerianum*, *A. obtusatum* subsp. *obtusatum*, *A. obtusatum* subsp. *northlandicum*, and *A. shuttleworthianum* (Table 1). From the molecular dating analysis above, the most recent common ancestor of all of these taxa is younger than the separation of New Zealand from Australia. Consequently, inference of at least one dispersal event for each of these taxa is required to explain their occurrence in New Zealand and elsewhere. Even leaving aside the molecular dating evidence above, it seems unlikely, as pointed out by Brownsey (2001), that the disjunct populations of each of these taxa have remained sufficiently unchanged morphologically to be regarded as the same taxon through some 80 m.y. of separation, if they were indeed vicariant.

Recent molecular studies have found long-distance dispersal to be prevalent in the origins of the New Zealand flora (reviewed by Winkworth *et al.*, 2002), with few exceptions (Stöckler *et al.*, 2002). Within ferns, molecular dating has suggested an origin via dispersal for New Zealand *Polystichum* (Perrie *et al.*, 2003a). In addition to receiving elements via long-distance dispersal, New Zealand has also acted as a source for other regions (Wright *et al.*, 2000; Lockhart *et al.*, 2001), and Brownsey (2001) suggested *Asplenium bulbiferum* subsp. *gracillimum* may have dispersed from New Zealand to Australia.

The Austral group of *Asplenium* is relatively widespread in the southern Pacific and Australasian regions. Conspecifics or close relatives of the New Zealand taxa from the Bulbiferum group are known to occur in Australia; the Flaccidum group in Australia and some south Pacific islands; and the Obtusatum group in Australia, South America and some south Pacific islands. Further sampling may indicate that species from outside the Australasian and southern Pacific regions, or other species present in these regions but not in New Zealand, may also belong to this Austral group.

Although each appears strongly circumscribed, the relationships among the Bulbiferum, Flaccidum, and Obtusatum groups are not well resolved because of one of the few points of conflict between the two molecular data sets studied

here. The *trnL-trnF* data strongly suggests that the Bulbiferum and Flaccidum groups are sister groups, whereas almost equally strongly the *rbcL* data suggests that the Bulbiferum and Obtusatum groups are each others' closest relatives. The underlying explanation for this conflict is not known, and it is likely that more character data will be required to resolve this issue. However, it can be noted that the highest frequency of inter-group hybridization is between the Bulbiferum and Flaccidum groups, with *Asplenium bulbiferum* subsp. *bulbiferum* × *A. flaccidum* subsp. *flaccidum* and *A. bulbiferum* subsp. *gracillimum* × *A. flaccidum* subsp. *flaccidum* being particularly common (Fig. 1).

Asplenium lamprophyllum, despite its creeping rhizome and broad lamina segments, falls within the Flaccidum group whose other constituents have erect rhizomes and narrow lamina segments. Also in the Flaccidum group is *A. shuttleworthianum*, which with its narrow lamina segments and marginal sori, bears a strong morphological resemblance to, and indeed can be difficult to separate from, the Pacific *A. gibberosum* (G.Forst.) Mett. The latter is sometimes placed (e.g., Brownlie, 1977) within the segregate genus *Loxoscaphe* T.Moore, as *L. gibberosum* (G.Forst.) T.Moore. However, *rbcL* sequence (Gastony and Johnson, 2001) from African material of the type species of *Loxoscaphe*, *L. thecifera* (Kunth) T.Moore (= *A. theciferum*), does not fall within the Austral group (Fig. 3). If *A. gibberosum*, for which sequence data is presently unavailable, is more closely related to *A. shuttleworthianum* than *A. theciferum*, then at least some authors' (e.g., Brownlie, 1977) circumscriptions of *Loxoscaphe* are polyphyletic. Alternatively, if *A. gibberosum* is more closely related to *A. theciferum* than *A. shuttleworthianum*, such that *Loxoscaphe sensu* Brownlie (1977) is monophyletic (albeit nested within *Asplenium*; Gastony and Johnson, 2001), then the resemblance of *A. shuttleworthianum* to *A. gibberosum* represents another case of striking morphological convergence within *Asplenium* (Murakami *et al.*, 1999a; Van den heede *et al.*, 2003).

Of the species investigated, the closest relative of *Asplenium pauperequitum* was found to be *A. flabellifolium*, and not *A. polyodon* as tentatively suggested by Brownsey and Jackson (1984). *Asplenium pauperequitum* is one of the rarest ferns in New Zealand, being known only from the Poor Knights Islands (Brownsey and Jackson, 1984). Nevertheless, the relationship between *A. flabellifolium* and *A. pauperequitum* is not close. These two species show little morphological similarity, and r8s, using the calibration described above, estimates a divergence time between them of 19 m.y. (with the caveats indicated above). Further sampling, particularly in the Pacific region, may find species with greater affinity to *A. pauperequitum*, and possibly even its tetraploid progenitor(s).

Brownsey (1977b) suggested that the octoploid *Asplenium appendiculatum* was probably derived from the tetraploid *A. flaccidum* via autopolyploidy. The discovery that the *trnL-trnF* sequence of *A. appendiculatum* subsp. *appendiculatum* is identical to that of *A. flaccidum* subsp. *haurakiense*, while the *trnL-trnF* sequence of *A. appendiculatum* subsp. *maritimum* is identical to those of *A. chathamense* and *A. flaccidum* subsp. *flaccidum* may indicate two independent polyploid events, mirroring other instances where different chlo-

roplast sequences have been found in both the putative progenitors and descendants (Soltis and Soltis, 1999). While certainly deserving of further investigation, the *trnL-trnF* sequences of *A. appendiculatum* subsp. *appendiculatum* and *A. flaccidum* subsp. *haurakiense* differ from the others only in having a thymine rather than an adenine at position 50. This base pair appears particularly prone to homoplasy, with reversion between an adenine and thymine occurring independently at least once in the Flaccidum group, at least twice in the Bulbiferum group, and again at least once outside the Austral group. The propensity for change shown by position 50 may be due to its central location in what appears, if transcribed, to be a hairpin loop-forming stretch of the *trnL-trnF* intergenic sequence. An indication of two independent polyploid origins for *A. appendiculatum* from these data is dependent on the associated change at position 50 not being homoplastic, which it clearly is in other instances.

The chloroplast sequence of *Asplenium shuttleworthianum* is quite distinct from any of the other New Zealand taxa sampled here from the *A. flaccidum* group. Given the primarily tropical distribution of *A. shuttleworthianum*, its maternal tetraploid progenitor, if still extant, is probably to be found in the Pacific.

Intriguingly, the *trnL-trnF* sequences from the octoploid *Asplenium bulbiferum* subsp. *gracillimum* implicate *A. hookerianum* as its chloroplast or maternal parent rather than *A. bulbiferum* subsp. *bulbiferum*. This raises the possibility that *A. bulbiferum* subsp. *gracillimum* is an allopolyploid between the non-bulbiferous *A. hookerianum*, from which it has inherited its chloroplast, and *A. bulbiferum* subsp. *bulbiferum*, with which it shares a close morphological similarity including bulbil production. This finding might be taken as support for the recognition at species level of the two entities here regarded as subspecies of *A. bulbiferum* (Ogle, 1987; Table 1), and this is presently being investigated further (Perrie and Brownsey, in prep.). The polymorphism shared between *A. bulbiferum* subsp. *gracillimum* and *A. hookerianum* is at position 50 which, because of the suspicion outlined above that this base pair is susceptible to homoplasy, should be interpreted cautiously in any inference of multiple polyploid events.

Asplenium cimmeriorum has an identical *trnL-trnF* sequence to one of the *A. bulbiferum* subsp. *gracillimum* samples. Whether the non-bulbiferous *A. cimmeriorum* is similarly derived from an allopolyploid event between *A. bulbiferum* subsp. *bulbiferum* and *A. hookerianum* – either directly via an independent event or following divergence from *A. bulbiferum* subsp. *gracillimum* – or is an autopolyploid of *A. hookerianum* requires further research of the sort employed by Perrie *et al.* (2003b) using genetic markers from throughout the genome. *Asplenium richardii* is sister to a clade that includes *A. hookerianum*, from which Brownsey (1977a) suggested the former was possibly an autopolyploid.

Brownsey (1977b) regarded *Asplenium lyallii* and *A. scleroprium* as probable allopolyploids based on their close morphological resemblance to sterile hybrids between members of the Bulbiferum and Obtusatum groups, and

Flaccidum and Obtusatum groups, respectively. The *trnL-trnF* sequences clearly implicate a chloroplast or maternal parent from the Obtusatum group for both *A. lyallii* and *A. scleroprium*. However, which of the two extant tetraploid species was involved in either case cannot be ascertained. Despite considerable differences in their spore morphology (Brownsey, 1977a), *A. oblongifolium* and *A. obtusatum* share identical sequences for both the *trnL-trnF* and *rbcL* markers. Their *psbC-trnS* sequences, another chloroplast intergenic spacer, are also identical (unpub. data). In contrast, the *trnL-trnF* sequence of *A. obtusatum* subsp. *northlandicum* exhibits two autapomorphies relative to *A. obtusatum* subsp. *obtusatum*, from which Brownsey (1977b) suggested the former arose by autopolyploidy.

New Zealand appears to have been colonized, via dispersal, by several major groups within *Asplenium*. Most of the New Zealand species, however, belong to the Austral sub-group of the Greater-nidus group, and their close relationship to one another is reflected in their ability to hybridize. Future work will involve obtaining sequence data from Australian and Pacific material to assess the relationships and migration patterns within *Asplenium* in this region. Investigation into the relationships amongst the *Asplenium* species in New Zealand will also continue, with an emphasis on understanding the evolutionary history of some of the polyploids.

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Literature Cited

Adachi, J. and M. Hasegawa. 1995. Phylogeny of whales: dependence of the inference on species sampling. Molec. Biol. Evol. 12:177–179.

Allan, H. H. 1961. *Flora of New Zealand, Vol. I.* Government Printer, Wellington, New Zealand.

Braithwaite, A. F. The *Asplenium aethiopicum* complex in South Africa. Bot. J. Linn. Soc. 93: 343–378.

Brownlie, G. 1977. The pteridophyte flora of Fiji. Beih. Nova Hedwigia 55:39–86.

Brownsey, P. J. 1977a. A taxonomic revision of the New Zealand species of *Asplenium*. New Zealand J. Bot. 15:39–86.

Brownsey, P. J. 1977b. *Asplenium* hybrids in the New Zealand flora. New Zealand J. Bot. 15: 601–637.

Brownsey, P. J. 1979. *Asplenium lucidum* Forst.f., an illegitimate name for the New Zealand shining spleenwort. New Zealand J. Bot. 17:217–218.

Brownsey, P. J. 2001. New Zealand's pteridophyte flora – plants of ancient lineage but recent arrival? Brittonia 53:284–303.

Brownsey, P. J. 2003. Recent pteridophytes. *In* D. Gordon [ed.], *The New Zealand inventory of biodiversity: a Species 2000 symposium review*. Canterbury University Press, Christchurch, New Zealand (in press).

BROWNSEY, P. J. and P. J. JACKSON. 1984. *Asplenium pauperequitum* – a new fern species from the Poor Knights Islands, New Zealand. New Zealand J. Bot. 22:315–321.

BROWNSEY, P. J. and P. J. DE LANGE. 1997. *Asplenium cimmeriorum*, a new fern species from New Zealand. New Zealand J. Bot. 35:283–292.

BROWNSEY, P. J. and J. C. SMITH-DODSWORTH. 2000. *New Zealand ferns and allied plants.* 2nd ed. David Bateman Ltd., Auckland, New Zealand.

DAWSON, M. I., P. J. BROWNSEY and J. D. LOVIS. 2000. Index of chromosome numbers of indigenous New Zealand pteridophytes. New Zealand J. Bot. 38:25–46.

DES MARIAS, D. L., A. R. SMITH, D. M. BRITTON and K. M. PRYER. 2003. Phylogenetic relationships and evolution of extant horsetails, *Equisetum*, based on chloroplast DNA sequence data (*rbcL* and *trnL-F*). Int. J. Plant Sci. 164:737–751.

DOYLE, J. J. and J. D. DOYLE. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.

GASTONY, G. J. and W. P. JOHNSON. 2001. Phylogenetic placement of *Loxoscaphe thecifera* (Aspleniaceae) and *Actiniopteris radiata* (Pteridaceae) based on analysis of *rbcL* nucleotide sequences. Amer. Fern J. 91:197–213.

HASEBE, M., T. OMORI, M. NAKAZAWA, T. SANO, M. KATO and K. IWATSUKI. 1994. *rbcL* gene sequence provide evidence for the evolutionary lineages of leptosporangiate ferns. Proc. Natl. Acad. Sc., USA. 91:5730–5734.

HOLMGREN, P. K., N. H. HOLMGREN and L. C. BARNETT. 1990. Index herbariorum. Part I: the herbaria of the world. 8th ed. Regnum Veg. 120:1–693.

HOOKER, J. D. 1855. *Flora Novae Zelandiae, Vol. II.* Reeve, London.

LOCKHART, P. J., P. A. MCLENACHAN, D. HAVELL, D. GLENNY, D. HUSON and U. JENSEN. 2001. Phylogeny, radiation and transoceanic dispersal of New Zealand alpine buttercups: molecular evidence under split decomposition. Ann. Missouri Bot. Gard. 88:458–477.

LÖVE, A., D. LÖVE and R. E. G. Pichi SERMOLLI. 1977. *Cytotaxonomical atlas of the Pteridophyta.* Cramer, Vaduz.

LOVIS, J. D. 1977. Evolutionary patterns and processes in ferns. Advances Bot. Res. 4:230–424.

MANTON, I. 1950. *Problems of cytology and evolution in the Pteridophyta.* Cambridge University Press, Cambridge.

MARTIN, W. 1920. Pteridophytes of Banks Peninsula (eastern portion). Trans. & Proc. New Zealand Inst. 52:315–322.

MCLOUGHLIN, S. 2001. The breakup history of Gondwana and its impact on pre-Cenozoic florisitic provincialism. Aust. J. Bot. 49:271–300.

MILDENHALL, D. C. 1980. New Zealand Late Cretaceous and Cenozoic plant biogeography: a contribution. Palaeogeogr. Palaeoclimatol. Palaeoecol. 31:197–233.

MURAKAMI, N. 1995. Systematics and evolutionary biology of the fern genus *Hymenasplenium* (Aspleniaceae). J. Pl. Res. 108:257–268.

MURAKAMI, N., S. NOGAMI, M. WATANABE and K. IWATSUKI. 1999a. Phylogeny of Aspleniaceae inferred from *rbcL* nucleotide sequences. Amer. Fern J. 89:232–243.

MURAKAMI, N., M. WATANABE, J. YOKOYAMA, Y. YATABE, H. IWASAKI and S. SERIZAWA. 1999b. Molecular taxonomic study and revision of the three Japanese species of *Asplenium* sect. *Thamnopteris*. J. Pl. Res. 112:15–25.

OGLE, C. C. 1987. Taxonomic changes in *Asplenium* (Aspleniaceae; Filicales) in New Zealand. New Zealand J. Bot. 25:591–593.

PERRIE, L. R., P. J. BROWNSEY, P. J. LOCKHART, E. A. BROWN and M. F. LARGE. 2003a. Biogeography of temperate Australasian *Polystichum* ferns as inferred from chloroplast sequence and AFLP. J. Biogeogr. 30:1729–1736.

PERRIE, L. R., P. J. BROWNSEY, P. J. LOCKHART and M. F. LARGE. 2003b. Evidence for an allopolyploid complex in New Zealand *Polystichum*. New Zealand J. Bot. 41:189–215.

PINTER, I., F. BAKKER, J. BARRETT, C. COX, M. GIBBY, S. HENDERSON, M. MORGAN-RICHARDS, F. RUMSEY, S. RUSSELL, S. TREWICK, H. SCHNEIDER and J. VOGEL. 2002. Phylogenetic and biosystematic relationships in four highly disjunct polyploid complexes in the subgenera *Ceterach* and *Phyllitis* in *Asplenium* (Aspleniaceae). Org. Divers. Evol. 2:299–311.

POSADA, D. and K. A. CRANDALL. 1998. Modeltest: testing the model of DNA substitution. Bioinformatics 14:817–818.

RAMBAUT, A. 1995. *Sequence Alignment Editor, v1.0a1*. (http://evolve.zoo.ox.ac.uk/software/Se-Al/main.html). Oxford University.

REICHSTEIN, T. 1981. Hybrids in European Aspleniaceae (Pteridophyta). Bot. Helvet. 91:89–139.

SANDERSON, M. J. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. Molec. Biol. Evol. 19:101–109.

SANDERSON, M. J. and J. A. DOYLE. 2001. Sources of error and confidence intervals in estimating the age of angiosperms from *rbcL* and 18S rDNA data. Amer. J. Bot. 88:1499–1516.

SCHULZE, G., J. TREUTLEIN and M. WINK. 2001. Phylogenetic relationships between *Asplenium bourgaei* (Boiss.) Milde and *A. jahandiezii* (Litard.) Rouy inferred from morphological characters and *rbcL* sequences. Pl. Biol. 3:364–371.

SKOG, J. E. 2001. Biogeography of Mesozoic leptosporangiate ferns related to extant ferns. Brittonia 53:236–269.

SOLTIS, D. E. and P. S. SOLTIS. 1999. Polyploidy: recurrent formation and genome evolution. Trends Ecol. Evol. 14:348–352.

STÖCKLER, K., I. L. DANIEL and P. J. LOCKHART. 2002. New Zealand kauri (*Agathis australis* (D. Don) Lindl., Araucariaceae) survives Oligocene drowning. Syst. Biol. 51:827–832.

SWOFFORD, D. L. 2002. *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4*. Sinauer Associates, Sunderland, Massachusetts.

TABERLET, P., L. GIELLY, G. PAUTOU and J. BOUVET. 1991. Universal primers for the amplification of three non-coding regions of the chloroplast DNA. Pl. Molec. Biol. 17:1105–1109.

TINDALE, M. D. and S. K. ROY. 2002. A cytotaxonomic survey of the Pteridophyta of Australia. Austral. Syst. Bot. 15:839–937.

THOMPSON, J. D., T. J. GIBSON, F. PLEWNIAK, F. JEANMOUGIN and D. G. HIGGINS. 1997. The CustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24:4876–4882.

VAN DEN HEEDE, C. J., PAJARÓN, E. PANGUA and R. L. L. VIANE. A new species and a new hybrid of *Asplenium* (Aspleniaceae) from Cyprus and evidence of their origin. Belg. J. Bot. 135:92–116.

VAN DEN HEEDE, C. J., R. L. L. VIANE and M. W. CHASE. 2003. Phylogenetic analysis of *Asplenium* subgenus *Ceterach* (Pteridophyta: Aspleniaceae) based on plastid and nuclear ribosomal ITS DNA sequences. Amer. J. Bot. 90:481–495.

VOGEL, J. C., S. J. RUSSELL, F. J. RUMSEY, J. A. BARRETT and M. GIBBY. 1998. Evidence for maternal transmission of chloroplast DNA in the genus *Asplenium* (Aspleniaceae, Pteridophyta). Bot. Acta 111:247–249.

WAGNER, W. H. 1954. Reticulate evolution in the Appalachian aspleniums. Evolution 8:103–118.

WIKSTRÖM, N. and P. KENRICK. 2001. Evolution of Lycopodiaceae (Lycopsida): estimating divergence times from *rbcL* gene sequence by use of nonparametric rate smoothing. Molec. Phylogen. Evol. 19:177–186.

WINKWORTH, R. C., S. J. WAGSTAFF, D. GLENNY and P. J. LOCKHART. 2002. Plant dispersal N.E.W.S from New Zealand. Trends Ecol. Evol. 17:514–520.

WRIGHT, S. D., C. G. YONG, J. W. DAWSON, D. J. WHITTAKER and R. C. GARDNER. 2000. Riding the ice age El Niño? Pacific biogeography and evolution of *Metrosideros* subg. *Metrosideros* (Myrtaceae) inferred from nuclear ribosomal DNA. Proc. Natl. Acad. Sc., USA. 97:4118–413.

YATABE, Y., S. MASUYAMA, D. DARNAEDI and N. MURAKAMI. 2001. Molecular systematics of the *Asplenium nidus* complex from Mt. Halimun National Park, Indonesia: evidence for reproductive isolation among three sympatric *rbcL* sequence types. Amer. J. Bot. 88:1517–1522.